8-1-2009

Searching for the Binding Partners for the Novel PHKG1 Variant γ 181

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SEARCHING FOR BINDING PARTNERS FOR THE NOVEL PHKG1 VARIANT γ181

A Thesis Presented to
The Faculty of the Department of Biology
Western Kentucky University
Bowling Green, Kentucky

In Partial Fulfillment
Of the Requirement for the Degree
Master of Science

By

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August 2009

SEARCHING FOR BINDING PARTNERS FOR THE NOVEL PHKG1

VARIANT γ181

Date Recommended 7/28/2009

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ACKNOWLEDGEMENTS

I express my deep sense of gratitude and respect to Dr. Nancy A. Rice, for her support, guidance and encouragement. Her critical evaluation and valuable suggestions during the course of this work have helped me immensely in gaining an insight into this subject. The scientific education I have received from WKU will help in pursuing higher in my career. Besides my advisor, I would like to thank the rest of my committee members, Dr. Claire Rinehart and Dr. Cheryl Davis; not only for their guidance but also for the educational enrichment they have provided me as well.

To the faculty and staff of the Department of Biology I would like to express my gratitude for their support. I want to thank Dr. John Andersland and the staff of the Biotechnology Center for their assistance. I am thankful to Dr. Bethel Sharma, with whom I was associated during every phase of this work. I would like thank other members of this lab, Clara, Joe, Ashley, Lauren, and Olivia. I would like to express my deepest gratitude to my friends, Raghu, Nanda, Sanjay, Lakshmi reddy, Bhagya for their support and friendship.

Last, but not least, I thank my parents and brother for their unconditional support and encouragement to pursue my interests.

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SEARCHING FOR BINDING PARTNERS FOR THE NOVEL PHKG1 VARIANT γ181

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Directed by: Nancy A. Rice, Claire A. Rinehart, Cheryl D. Davis

Department of Biology Western Kentucky University

Phosphorylase kinase (Phk), a key regulatory enzyme in glycogenolysis, is a hexadecameric protein that contains four different subunits with a stoichiometry $(\alpha\beta\gamma\delta)_4$. Alpha and β are regulatory subunits, γ is catalytic and δ is an intrinsic molecule of calmodulin. The gene that encodes for the γ subunit PHKG1 undergoes alternative processing to produce a truncated form of γ containing 181 residues (γ181) instead of the normal 387 residues. Although much of the kinase helical domain is absent, γ181 is still catalytic with its activity being influenced by PKC, and its mRNA being expressed in high levels in brain. S peptide is the only known *in vitro* substrate for γ181. In the present study, we are using the LexA-based yeast two-hybrid system to identify binding partners for γ181. Gamma181 was cloned into the pLexA vector and used as bait to screen a human fetal brain cDNA library. Interactions between γ181 and cDNA library proteins were monitored by auxotropic growth of the yeast in the absence of leucine and by expression of β-galactosidase activity. Following screening for leucine prototrophy and β-galactosidase activity, we obtained 37 colonies that show positive for leucine prototrophy, although only 20 of these colonies were β-galactosidase positive. Among these 20 colonies, 7 unique clones were identified corresponding to the proteins RPL23, RPL9, and NA14. The physiological relevance of these interactions in the brain is yet to

be discovered.

Introduction

A. Phosphorylase kinase

Phosphorylase kinase (Phk), the first protein kinase discovered, belongs to the serine/threonine kinase super family. Phk, a key regulatory enzyme of glycogenolysis, is a hexadecameric protein that contains four different subunits with a stoichiometry of $(\alpha\beta\gamma\delta)_4$ and molecular mass of $1.3x10^6$ Da (Brushia and Walsh, 1999; Cohen, 1973). Alpha and β are regulatory subunits, γ is catalytic (Skuster et al., 1980) and δ is an intrinsic molecule of calmodulin which binds tightly to the holoenzyme (Cohen et al., 1978; Grand et al., 1981).

Phosphorylase kinase has a vast diversity of expression levels in different tissues with liver and muscle having higher amounts. Determination of the spatial expression pattern of the Phk subunits by Expressed Sequenced Tag (EST) and UniGene database analysis revealed that Phk may be expressed in 28-36 different tissues (Winchester et al., 2007). In muscle cells Phk exists in two different compartments. The bulk of the enzyme is located in the cytoplasm and is associated with the glycogen particles (Meyer et al., 1970). Studies on localization of phosphorylase kinase revealed that a fraction of the Phk is directly associated with the membranes of T-tubules and sarcoplasmic reticulum of muscle cells (Thieleczek et al., 1987 and Dombradi et al., 1984). Farnesylation of the α and β subunits of Phk provided additional evidence for association of Phk with the membrane in muscle cells (Heilmeyer et al., 1992). Even though all of the enzyme molecules get farnesylated, only a small amount of Phk appears to be associated with the membranes.

Structurally, Phk is a complex enzyme made up of 16 subunits. Three dimensional structural studies of the Phk by cryo-electron microscopy (Bryan et al., 2009), negative staining, and scanning transmission electron microscopy (Trempe et al., 1986) reported a bilobal form of the enzyme also know as the butterfly like view (Fig.1.1). Eighty percent of the total mass of the enzyme is composed of the α and β subunits, thus α and β are the major contributors to the overall 3-D structure of Phk.

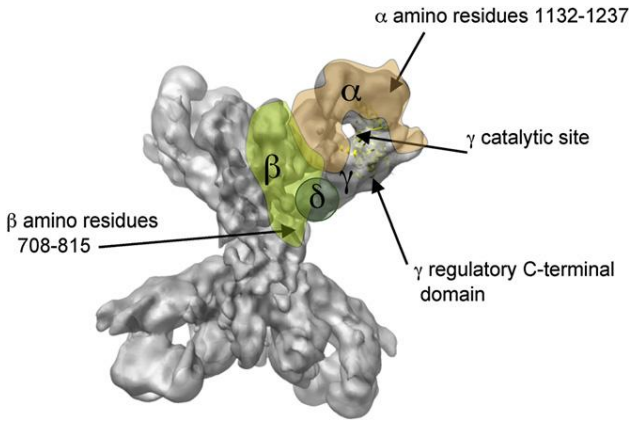

Fig. 1.1. Schematic location of the four subunits in the 3-D Cryo-EM volume of phosphorylase kinase holoenzyme (Venien-Bryan et al., 2009).

In the butterfly like structure, Phk has two lobes which are connected at the center of the lobe by two bridges. The α subunit of Phk is located at the wing tips of the butterfly orientation of the holoenzyme (Wilkinson D.A., 1994). Proteolytic degradation of the β subunit by trypsin disrupts the butterfly structure of the holoenzyme whereas proteolysis of α subunit has little effect on the structure of Phk. This supports the idea that β is required to maintain the integrity of the structure, and it forms the bridge between the two lobes (Trempe et al., 1986). Phk protein crosslinking results in the formation of ββ dimers which further supports that each bridge in the holoenzyme was formed by the ββ dimer (Ayers et al., 1998).

The α as well as β subunits of Phk are well known for their regulatory roles in the holoenzyme. The α subunit has 1237 amino acids with a molecular mass of 138 KDa (Zander et al., 1988). There are two separate genes encoding the two isoforms of the α subunit, and a single gene encoding the multiple isoforms of the β subunit. Additionally, two forms of the muscle specific α subunit exist as a result of alternative splicing of the PHKA1 gene; the truncated form lacks 59 amino acids in the middle of the polypeptide (Harmann et al., 1991). In the nonactivated holoenzyme, quaternary interactions of the α and β subunits inhibit the catalytic activity of the γ subunit (Paudel and Carlson, 1987). The presence of highly conserved glucoamylase like domains in the α and β subunits suggests a functional role for these subunits in release of glucose from the nonreducing ends of glycogen in addition to the regulation of the Phk holoenzyme (Pallen, 2003). Extrinsic calmodulin, also known as δ', also activates the Phk by directly binding to the α and β subunits. This activation purely depends on the presence of calcium. The δ' subunit will not activate the tryptic and chymotryptic digests of Phk which provides evidence for interaction of the δ' subunit with the α, β subunits (Picton et al., 1980).

The β subunit maintains the structural integrity of the holoenzyme (Trempe et al., 1986). The β subunit consists of 1029 amino acid residues and has two serine phosphorylation sites, β-26 and β-700 that are good substrates for protein kinase A (Heilmeyer, 1991). Residues 420-436 of the β subunit contain a region that has sequence homology with the natural substrate glycogen phosphorylase b (Pb) which acts as an auto inhibitor and regulates the activity of the γ subunit (Sanchez and Carlson, 1993). Phosphorylation and farnesylation are the two post translational modifications that occur

on the α and β subunits. The exact function of farnesylation of the α and β subunits *in vivo* is unknown. A cluster of 7 phosphorylation sites (aka multiphosphorylation domain) was identified in the α subunit.

The γ subunit of phosphorylase kinase has a molecular mass of 41,000 daltons (Cohen, 1973) and harbors the catalytic site. The N-terminus of the γ subunit (amino acids 1-298) shares extensive sequence homology with the catalytic domain of most kinases, whereas the C-terminal region (last 110 amino acids) shows sequence homology with the calmodulin dependent kinase troponin I.

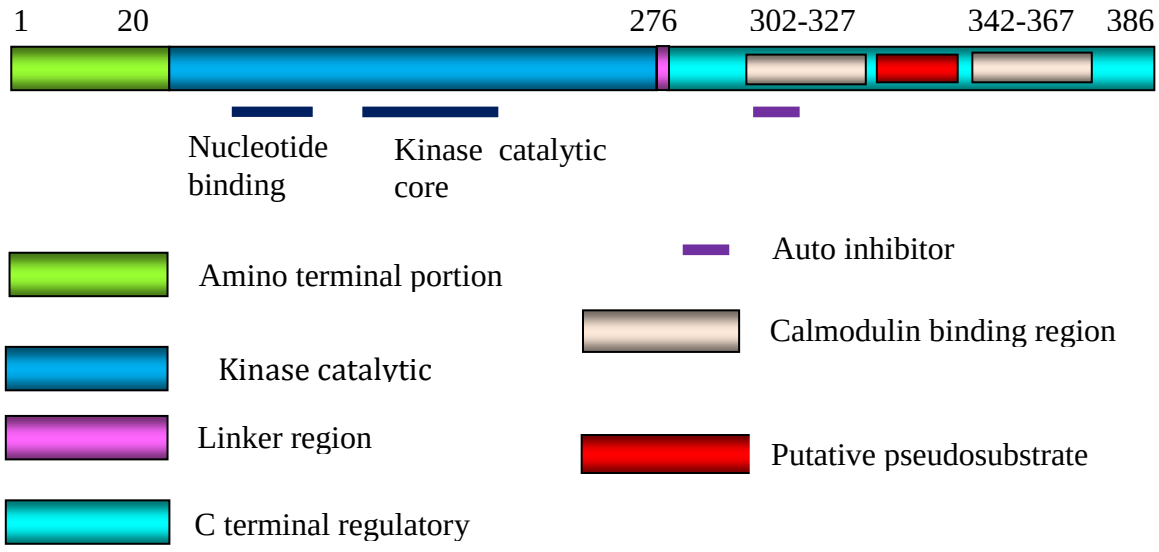

Fig. 1.2. Schematic representation of the γ subunit of phosphorylase kinase (Owen et al., 1995).

The C-terminus of the γ subunit mediates the interaction with the δ and α subunits. The γ subunit has two noncontiguous calmodulin binding sites, γ302-326 and γ342-366. Both of these are rich in positively charged amino acids and bind to the δ subunit

simultaneously (Dasgupta et al., 1989). Electrostatic interactions play an important role in the interaction between the γ and δ subunits because there is a drastic reduction in the activation of the γ subunit by the δ subunit when the acid residues in the E helices of the δ subunit are changed to basic residues (Farrar et al., 1993). The C-terminal region of the γ subunit (residues 302-312) acts as an auto inhibitory region. *In vitro* studies with a peptide corresponding to amino acids 302-312 have shown that this peptide binds to the catalytic site of a truncated γ subunit (1-300) and acts as an inhibitor. This peptide called Phk13-1, also decreases the ATPase activity of the truncated γ (1-300) (Bartleson & Graves., 2001). Yeast two-hybrid and protein crosslinking studies by Rice et al., (2002) showed the C-terminus of the α subunit (amino acids1060-1237) interacts with the C-terminus of the γ subunit (amino acids 343-386). Furthermore, there is an increase in αγ heterodimer formation in the presence of Ca^{2+} which reveals that Ca^{2+}indirectly mediates changes in the interaction between the α and γ subunits (Rice et al., 2002). Studies with a purified γ subunit show high catalytic activity even in presence of EGTA, suggesting that Ca^{2+} is not required for the purified γ subunit of Phk to maintain its activity (Skuster et al., 1980). The purified γ subunit also phosphorylates well-known *in vitro* substrates of Phk such as myelin basic protein, k-casein, skeletal muscle troponin complex and troponin T. Free Mg^{2+} activates the γ subunit whereas free Mn^{2+} inhibits the activity (Kee and Graves, 1987). By using two different database searches Winchester et al., (2007) showed that the α, β, and γ subunits undergo extensive posttranscriptional processing. The PHKG1 gene which encodes the γ subunit of Phk, undergoes alternative cleavage and polyadenylation in intron 6 which results in the formation of a truncated

form of the γ subunit resulting in 181 amino acids instead of the normal 387 amino acids. The transcriptional expression level of this truncated form of γ is proposed to be high in the brain (Winchester et al., 2007).

The δ subunit of Phk consists of a single polypeptide of 148 amino acids with a molecular weight of 16,680 daltons (Grand et al., 1981). It is an intrinsic molecule of calmodulin that confers the Ca^{2+} sensitivity to Phk, and it associates tightly with the γ subunit even in the absence of Ca^{2+} (Picton et al., 1980). While unusual, this is not an exceptional case because it has been shown that adenylate cyclase from the bacterium *B. pertusis* has two calmodulin binding domains and associates with calmodulin even in the absence of Ca^{2+}, which helps to protect the adenylate cyclase from proteolytic degradation (Ladant, 1988). In addition to this, the δ subunit also provides a stabilizing role in Phk and prevents the aggregation of the α, β, γ and δ subunits in the holoenzyme thus confining the number of protomers of Phk (Paudel & Carlson, 1990). It also plays a dual role in the holoenzyme. In the absence of calcium, it inhibits Phk activity, and in the presence of calcium it activates Phk (Hessova et al., 1985) suggesting a tight association of the δ subunit with the enzyme even in the absence of calcium. Purified δ subunit, as well as the holoenzyme, activates phosphodiesterase and myosin light chain kinase in the presence of calcium and confers calcium sensitivity to the holoenzyme is provided by the δ subunit.

Glycogen phosphorylase b (Pb) is the only known *in vivo* substrate for Phk, even though myelin basic protein, k-casein, and skeletal muscle troponin complex and troponin T are known to be phosphorylated by Phk *in vitro* (Pickett-Gies and walsh, 1986 a). Recent studies by Boulatnikov et al., (2008) showed that glyceraldehyde3-phosphate

dehydrogenase (G-3DH), an intermediatory enzyme in glycolysis, is also phosphorylated by Phk, and its phosphorylation activity depends upon $Ca^{2+}$and pH. Interaction of G-3DH with Phk provides evidence for the direct link between glycogenolysis and glycolysis (Boulatnikov et al., 2008).

Regulation of Phk in relation to its function is a highly complex process. Phk activity is well regulated by the extracellular hormone epinephrine and also Ca^{2+}. Epinephrine is known to activate glycogenolysis in skeletal muscle. The downstream targets in the epinephrine signaling pathway are protein kinase A, Phk, and Pb (Yeaman and Cohen, 1975). Epinephrine triggers the activation of Pb thus providing the energy for the cell to meet the requirements of muscle contraction. The holoenzyme itself is regulated by the autoinhibitor sequences present in the β and γ subunits which bind to the catalytic site resulting in inhibition of the phosphotransferase activity. Phosphorylation of both the α and β subunits by cAMP dependent kinases regulates the phosphotransferase activity of Phk (Ramachandran et al., 1987). Even though there is no evidence for autophosphorylation of Phk *in vivo, in vitro* studies showed that autophosphorylation of the regulatory subunits by Phk itself increases its specific activity (Hallenbeck and Walsh, 1983). *In vitro* studies have revealed that the phosphotransferase activity of the Phk holoenzyme is highly dependent on pH changes, whereas the purified $\gamma\delta$ complex is less dependent. With an increase in pH from 6.8 to 8.2, the activity also increases (Chan and Graves, 1982). The exact mechanism of how increases in pH relieve the inhibition of the holoenzyme by the regulatory subunits is not known.

As previously discussed, Ca^{+2} is also known to be a major regulator of the

phosphotransferase activity of Phk. Calcium enhances the activity of the enzyme by directly binding to the δ subunit. Extrinsic calmodulin, δ' is also known to activate (Pickett-Gies and Walsh, 1986 b) the enzyme by binding to the α and β subunits (Picton et al., 1980). Both extrinsic and intrinsic calmodulin molecules depend upon on $Ca^{2+}$for Phk activation. Limited proteolysis of the α and β subunits by trypsin raises the enzyme activity at pH 6.8 (Cohen, 1973).

B. Protein-protein interactions and the Yeast Two-Hybrid System

Protein-protein interactions are a fundamental phenomenon for majority of cellular function. The majority of biological processes such as DNA replication, transcription, translation, and splicing depend on the interaction between specific proteins in the cell. Several enzymes including hemoglobin, the pyruvate dehydrogenase complex, Phk, and RNA polymerase all have multiple subunits. Interactions among the individual subunits are highly specific and are very important for the enzymatic functions and regulation. In addition to this, several transient protein-protein interactions also exist within the cells which regulate processes such as signal transduction, metabolic pathways, and cell cycle regulation. Transient protein-protein interactions are observed between protein modifying enzymes such as kinases, phosphatases, glycosylases, and their substrates. Interaction between proteins or among the subunits of enzymes has several effects within the cell. One such major effect of protein-protein interactions is dynamic changes in the kinetic properties of the enzymes. For example, in the nonactivated holoenzyme, quaternary interactions of the α and β subunits inhibit the

catalytic activity of the γ subunit of Phk (Paudel and Carlson, 1987).

Sequencing the human genome and assigning function to the newly identified gene products is one of the biggest challenges for biologists today. One of the best ways to determine function of newly identified gene products is to study the protein with which it is interacting in the cell. Therefore, understanding protein-protein interactions that occur within the cell is very crucial. Several biochemical and genetic methods have been developed to study protein-protein interactions. Commonly used biochemical techniques to study protein-protein interactions *in vitro* are co-immunoprecipitation, cross linking, and affinity blotting. Genetic methods such as the yeast two-hybrid system and phage display are also widely used to study protein-protein interactions *in vivo* (for review see Phizicky and Fields, 1995). Extensive research on the eukaryotic transcription factor Gal4 was a breakthrough for the development of the yeast two-hybrid system. Gal genes are required for the growth of the yeast cells on galactose medium, and consist of the Gal 1, 7, 2, 10 structural genes and the Gal 3, 4, 80 regulatory genes. Gal4 is an important transcriptional activator that regulates the transcription of the Gal 1, 7, 10 structural genes by binding to the upstream activator sequences (for review see Traven et al., 2006). The Gal4 transcriptional activator has a DNA binding domain and an activation domain; these two domains can be separated from each other physically and functionally. This was shown by fusing the β-galactosidase encoding lacZ gene to the Gal4 DNA binding region followed by the detection of the gal4- β-galactosidase fusion protein in yeast cells. The fusion protein was localized to the nucleus. Silver et al., (1984) also showed that only the 74 N- terminal amino acids are required for sufficient nuclear localization of the gal4-β-galactosidase fusion protein. The N-terminal 1-98 amino acid region of this DNA

binding (BD) domain alone will not activate the transcription (Keegan et al., 1986).

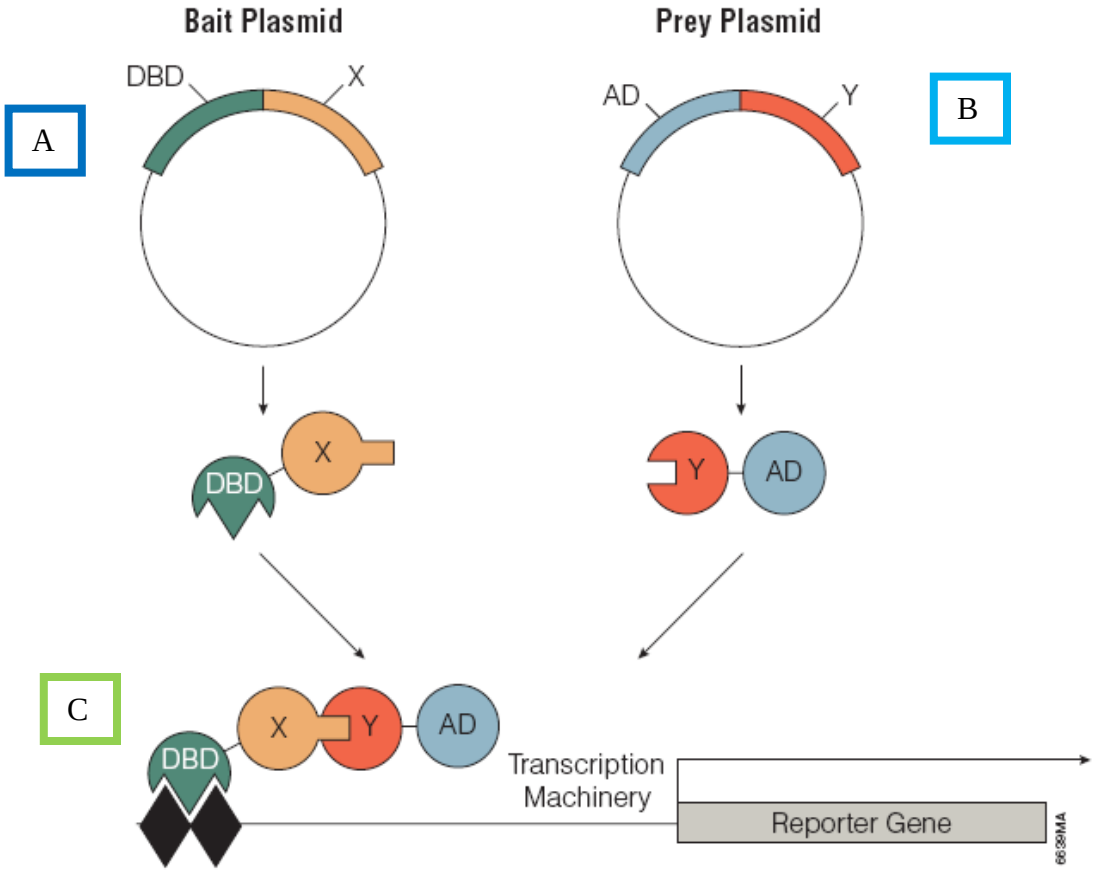

Fig. 1.3 Schematic representation of the yeast two-hybrid system. A. The coding sequence of bait protein (X) is cloned into BD containing vector which expresses BD-bait fusion protein B. Coding sequence of prey protein is cloned into an activation domain containing vector which express the AD-prey fusion protein C. Interaction of protein X with protein Y results in the reconstitution of the transcription factor and expression of the reporter gene. (Promega protein interaction guide, Chapter 1, yeast two-hybrid system, pp 1-3).

The functional activity of the separated DNA binding (BD) and activation domain (AD) of the Gal4 transcription factor was also later demonstrated by constructing the Gal80-AD fusion protein which was able to activate the transcription of downstream targets that are under the control of UAS_G by interacting with the Gal4 transcription factor. Thus, the BD and AD do not need to be in the same polypeptide for them to function (Ma & Ptashne, 1988). If the BD and AD are physically separated and they do not interact with each other, they will not activate transcription of the genes. However, if the BD and AD are physically close to each other, they will activate transcription. This was first shown by Fields and Song (1989). In their work, the Gal4 BD and the Gal4 AD were fused to the well characterized interacting proteins SNF1 and SNF4, respectively, and then transferred into yeast cells. Interaction between these two proteins, SNF1 and SNF4, resulted in the BD and AD being physically close to each other. Positive transcriptional activation was measured by β-galactosidase activity of the Gal1-β-galactosidase fusion protein (Fields and Song, 1989).

The yeast two-hybrid system has advantages over classical biochemical techniques such as co-immuno precipitation, cross linking, and affinity blotting. One of the major advantages is that it uses yeast cells as host cells, which have greater resemblance to higher eukaryotes. Also, transient interactions that occur in the cell are relatively weak, and such weak interactions can be detected in the yeast two-hybrid system. By using the yeast two-hybrid system the strength of interaction between the interacting proteins can also be measured (Sobhanifar, 2003). By using this system, any protein of interest termed a “bait” protein, can be screened against library proteins fused to AD to identify new binding partners for the bait (Chien et al., 1991).

Even though the yeast two-hybrid system is extensively used to study protein-protein interactions, it has some limitations. Posttranslational modification such as glycosylation occurs in the endoplasmic reticulum. As all the fusion proteins are targeted to the nucleus, interactions that depend upon post translational modifications cannot be detected in the yeast two-hybrid system. Thus, it has limitations for the use in detecting associations with extracellular and membrane associated proteins (Allen et al., 1995). Also, fusion proteins which are used in the two-hybrid system may change the conformation of the bait or prey, resulting in alteration of the binding activity of the proteins (Sobhanifar, 2003).

The first two-hybrid method developed was based on the Gal4 transcription factor (Fields and Song, 1989). In the Gal4 dependent yeast two-hybrid system, the yeast strain used for this study carried a deletion of the Gal4 and Gal80 genes, and grew more slowly than the wild-type strain. In order to avoid such problems, the LexA based method, also known as "interaction trap", was developed (Golemis et al., 2008). LexA is a repressor protein (Ebina et al., 1983) that represses the transcription of the several SOS genes in *E.coli*. Brent and Ptashne (1984) showed that the LexA protein expressed in yeast cells was passively transported into the nucleus and repressed the transcription of Gal1 promoter derivatives containing at least two LexA operators.

In the LexA-based system, nutritional markers used for selection are different from other two-hybrid systems. The bait plasmid contains the LexA DNA BD, and it is maintained in the cells by the presence of the *HIS3* gene which confers histidine prototrophy to the cells. The prey plasmid contains the B42 AD, and cDNA is inserted into the prey plasmid so that it will express cDNA-B42 AD fusion proteins. The prey

plasmid is maintained by the presence of the *TRP1* gene conferring the ability to grow in the absence of tryptophan. The AD fusion protein produced in the cell is under the control of the *GAL1* promoter, whose expression is induced by growing the cells in medium which contains galactose/raffinose. AD fusion protein levels are tightly regulated by this inducible promoter. This kind of regulation allows the cells to protect the normal cellular functions from the toxic fusion proteins. A low background LexAoperator-*LEU* reporter gene which is located on the chromosomal DNA is used to monitor the protein-protein interactions. It has six LexA operators upstream of the *LEU2* gene. The second reporter gene LexAoperator-*LacZ* is maintained on the *URA3* (uracil prototrophy) based plasmid. This plasmid has eight LexA operators upstream of the LacZ gene (Bartel and Fields, 1997). This kind of dual selection method makes the yeast two-hybrid system more reliable by reducing the number of false positives (Allen et al, 1995). The LexA repressor tagged bait fusion protein potentially reduces auto activation of the reporter genes (Allen et al., 1995).

Protein-protein interactions are monitored *in vivo* by leucine prototrophy and LacZ expression. The second reporter gene monitors LacZ gene expression in the cells which makes the β-galactosidase enzyme. Its activity can be measured by using different substrates. The most commonly used substrate is 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal). Beta-galactosidase converts the colorless substrate, X-Gal, into a blue color product. A more sensitive β-galactosidase assay uses the substrates ONPG and 1, 2 dioxetane which allow for measurement of the strength of the interactions quantitatively (Gietz et al., 1997).

C. Rationale of the project

Phk is a highly complex enzyme consisting of 16 protomers. Regulation of the phosphotransferase activity of Phk is a highly complex process. Extensive research has been carried out to understand the interaction of the protomers of the Phk and their role in the regulation of phosphotransferase activity. Understanding the spatial distribution of Phk is also very important. Winchester et al., (2007) showed by using EST and Uni Gene databases that Phk is expressed in nearly 28-36 different tissues, and that the α, β and γ subunits undergo extensive alternative transcriptional processing. In addition, they also found that there is a weak polyadenylation signal in intron6 of the gene which encodes for the γ subunit, PHKG1. An intronic polyadenylation signal will allow for alternative processing of the mRNA corresponding to the γ subunit. *In silico* translation of the alternatively processed mRNA resulted in a γ subunit with 181 amino acids (γ181) followed by an additional 21 amino acids which are derived from the intronic region. These unique C terminal 21 amino acids are highly conserved among primates. Biochemical evidence also showed that γ181 mRNA is expressed in the brain, heart, skeletal muscle, and tongue with brain having the higher levels (Winchester et al., 2007). The truncated form of the γ subunit lacks residue E 182 which determines the specificity of the enzyme for the Phb substrate. Furthermore the prosite database revealed that the unique 21 amino acids at the C-terminus of γ181 has a putative protein kinase C (PK-C) phosphorylation site. Surprisingly, despite being truncated, recombinantly expressed γ181 retains its kinase activity toward a peptide substrate, and this activity depends upon phosphorylation of γ181 by PK-C (Unpublished data). *In vivo* substrates are currently not known for the γ181 subunit. In this study we have used the LexA based yeast two-hybrid

system to identify potential binding partners for γ181 in the brain.

MATERIALS AND METHODS

A. Yeast and bacterial strains

The yeast strain used for the yeast two-hybrid screening was EGY 48 (MAT a, his3, trp1, ura3, 6ops-Leu2) (Clontech, Mountain View, CA). All plasmid manipulations were carried out in *E. coli* strain DH5α. The brain cDNA library was amplified in the high efficiency transformation strain DH10B (MegaX DH10B™ $T1^R$ Electrocomp™ Cells, Invitrogen). All of the library plasmids were rescued in the *E. coli* strain KC-8 (*hsdR, leuB600, trpC9830, pyrF*::*Tn5, hisB463, lac*Δ*X74, strA, galU,K*).

B. Two hybrid plasmids

pLexA, pB42, pJK101, and p8op-LacZ plasmids were used for this study (Table 1). All of the basic plasmids were obtained from Clontech (Clontech, Mountain View, CA). The pLexA basic vector (Fig. 2.1) was used for cloning the coding sequence of the bait protein. Other constructs cloned into the pLexA vector were p53, and the *Drosophila* bicoid protein coding sequence. The ADH1 promoter controls the expression of LexA fusion proteins. HIS3 is the marker used for selection in the yeast. The pB42 AD vector was used for cloning the cDNA library. A human fetal brain cDNA library cloned into the pB42 vector (Fig. 2.2) was kindly provided by Dr. Erica Golemis of Fox Chase Cancer Center, Philadelphia, PA. The GAL1 promoter controls the expression of the B42-cDNA cassette. The cassette also included a nuclear localization signal for targeting the B42-prey fusion proteins to the nucleus and a hemagglutinin epitope tag for assessment of the size of the encoded protein. This vector also has a TRP1 marker for

selection in the yeast cells. The p8op-LacZ plasmid (Fig. 2.3) is the most sensitive reporter plasmid used in the LexA based two-hybrid system. It has eight LexA operators followed by the LacZ reporter gene. It has a URA3 marker for selection in yeast cells. Other plasmids used in this study include, pJK101, pRFHM1, and pLexA-POS plasmids which were used for the repression assay. The pJK101 plasmid has an UAS_G followed by the LexA operator sequence followed by LacZ reporter gene. It has URA3 marker for selection in the yeast cells. pRFHM1 expresses LexA-bicoid fusion proteins used as a positive control in the repression assay. pLexA-POS expresses a LexA-GAL4AD fusion protein, and was used as negative control in the repression assay. Both pRFHM1 and pLexA-POS have a HIS3 marker for selection in the yeast cells. Transcriptional repression of the LacZ gene by LexA-γ181 was compared with the negative control LexA-bicoid protein. All of the plasmids used in this study have an ampicillin marker for selection in bacteria (Bartel & Fields, 1997).

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Table 1: Plasmids used in this study

Plasmid	Marker	Characteristic features
PLexA	*His3*, Amp^R	Has a LexA BD followed by the polylinker under the control of ADH1 constitutive promoter. Used for cloning the coding sequence of the bait protein.
PB42-empty	*Trp1*, Amp^R	Gal1 promoter expresses nuclear localization domain, transcriptional activation domain, HA epitope tag. Used for cloning the cDNA library.
p 8op-LacZ	*Ura3*, Amp^R	Used as a sensitive reporter plasmid in the LexA based two-hybrid system. It has eight LexA operators followed by the lacZ reporter gene.
pLexA-pos	*His3*, Amp^R	Expresses LexA-Gal4p fusion protein. Expresses LexA-Gal4AD fusion protein, used as negative control for repression assay.
pRFHM1	*His3*, Amp^R	Expresses LexA-bicoid fusion protein. Used as a positive control for repression assay.
pJK101	*Ura3*, Amp^R	Has a UAS_G followed by two LexA operators followed by lacz gene. Used in the repression assay for the detection of the nuclear localization of the LexA fusion proteins.
pLexA-γ181	His, Amp	Expresses LexA-γ181 fusion protein
pB42-cDNA	Trp, Amp	Expresses B42-cDNA fusion proteins

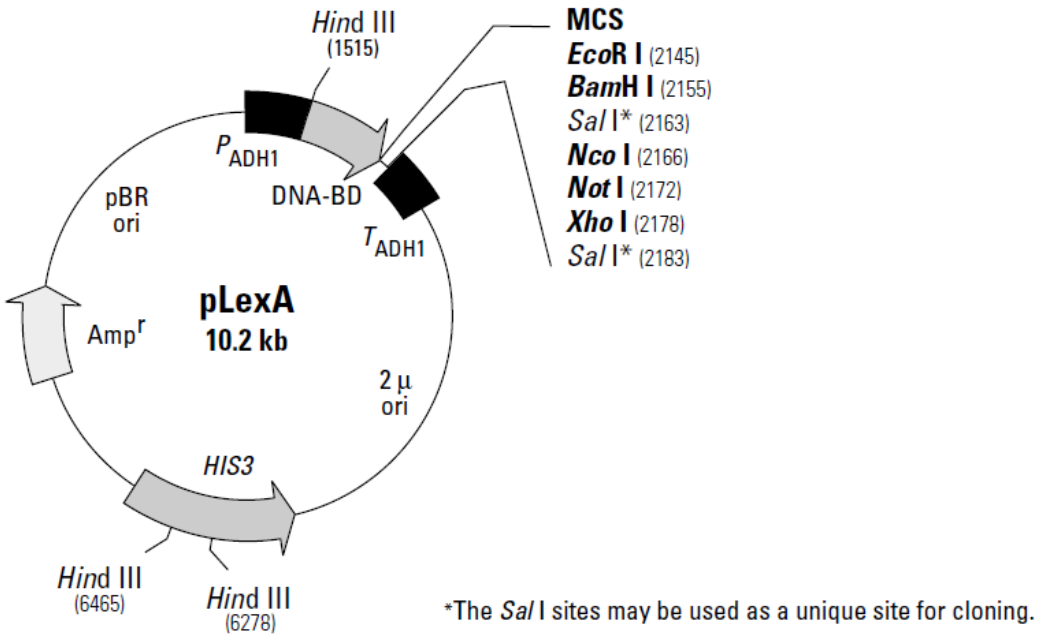

Fig. 2.1 pLexA vector. The human cDNA sequence encoding γ181 was cloned into multiple cloning site (MCS) of the pLexA vector to use as bait in yeast two-hybrid screening. It has a LexA DNA BD and a constitutive *ADH1* promoter that regulate the expression of LexA fusion proteins. *HIS3* is used as a marker for selection in yeast (Gyuris et al., 1993). The ampicillin resistance gene, Amp^r and the pBR origin allows selection and replication in *E.coli*. The 2 µ plasmid origin allows replication in yeast.

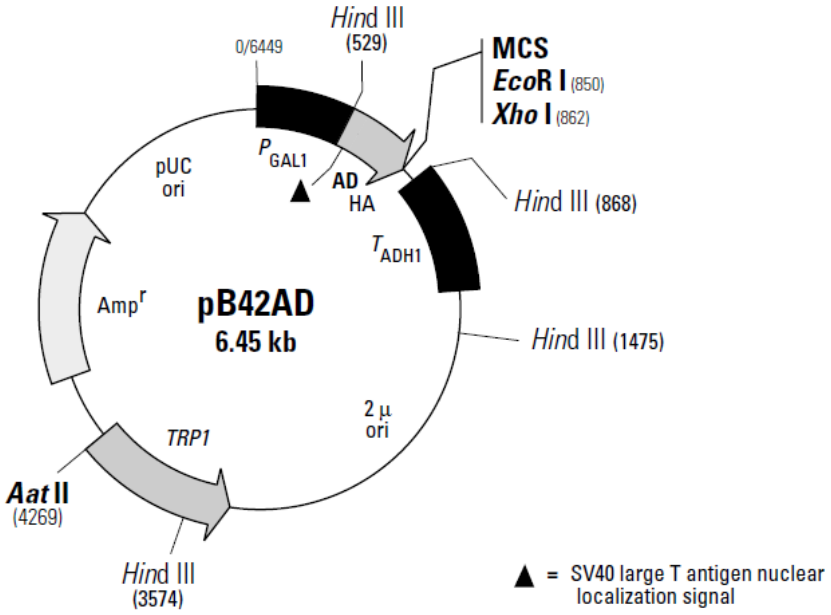

Fig. 2.2 pB42AD vector. The human fetal brain cDNA library was cloned into the MCS of the pB42 vector to use as prey in the yeast two hybrid screening. It has a B42 AD and the *GAL1* inducible promoter regulates expression of cDNA-B42 fusion proteins. The transcriptional terminator is derived from ADH1. A hemagglutinin epitop tag (HA) and a nuclear localization signal are also present on the plasmid. *TRP1* is used as a marker for selection in yeast (Gyuris et al., 1993). The ampicillin resistance gene, Amp^r and the pBR origin allows selection and replication in *E.coli*. The 2 µ plasmid origin allows replication in yeast.

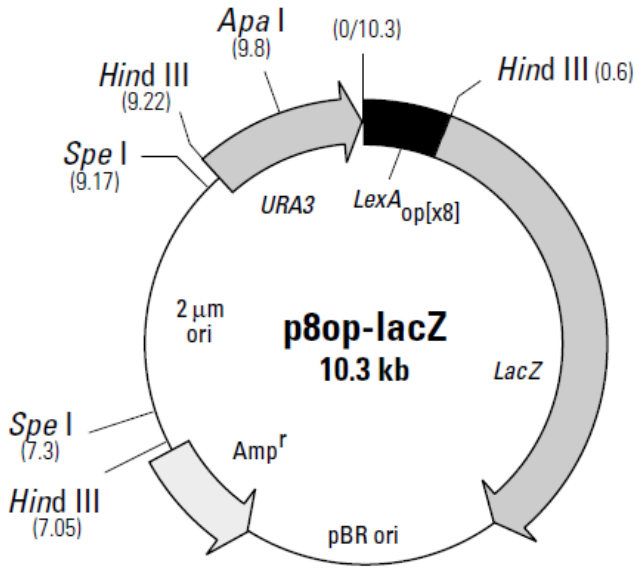

Fig. 2.3 p8op-LacZ plasmid. The LacZ gene is used as reporter of β-galactosidase activity. The LacZ gene is under the control of 8 lexA operators and a minimal TATA region form the *GAL1* promoter. *URA3* is used as a marker for selection in yeast (Estojak., 1995). The ampicillin resistance gene, Amp^r and the pBR origin allows selection and replication in *E.coli*. The 2 µ plasmid origin allows replication in yeast. The ampicillin resistance gene, Amp^r and the pBR origin allows selection and replication in *E.coli*. The 2 µ plasmid origin allows replication in yeast.

C. Cloning of γ181 into the pLexA vector

Human cDNA corresponding to the sequence of γ181 gene was synthesized by Genescript (Genescript, Piscataway, NJ) and cloned into pUC57 with optimized codon usage in *E. coli*. pUC57-γ181 was used as the template for PCR amplification of γ181 using the following primer pairs: gamma forward (ATCCGGAATTCATGACCCGGGACGA) and gamma reverse (ATCGCGGATCCCTATTCTGCAGATGG). Both primers contain restriction sites for EcoRI and BamH1, which allowed the amplicon to be digested with restriction enzymes EcoRI and BamHI (New England Biolabs) and ligated into precut pLexA with the same restriction sites. The PCR reaction was carried out in a Techne TC-512 thermocycler for 25 cycles under the following conditions: initial denaturation at 94 ^{o}C for 1min, denaturation at 94 ^{o}C for 1min, annealing at 60 ^{o}C for 2min, extension at 72 ^{o}C for 1min, final extension at 72 ^{o}C for 5min. The insert and vector were combined in a 10:1 volume ratio with T4 DNA ligase (Takara ligation kit) to carry out the ligation. The ligation mix was incubated at 16 ^{o}C for 4 hrs. After incubation, the ligation mixture was directly transformed into DH5α chemically competent cells by heatshock at 42 ^{o}C. Positive clones were selected by plating the transformants on LB plates containing 100 μg/ml of ampicillin.

D. Yeast transformation

EGY 48 cells containing the p8op-LacZ plasmid were made competent by growing 5 ml of overnight culture in synthetic media (SD) lacking uracil (SD-URA) medium (Clonetech, Mountain View, CA) followed by subculturing into 300 ml of the

same media. Subcultured cells were grown for 2-3 hours until mid log phase or an optical density (OD) 0.4-0.5nm. Cells were resuspended in 1.5 ml of 0.1 M lithium acetate (LiAc), 1M Tris-EDTA (TE) buffer and then shaken at 250 rpm for 30 min at 30 ^{o}C. These competent cells were then used for the transformation of the bait and library plasmids. The transformation mix was prepared by combining 1 μg of plasmid DNA, 10 μl of 10 mg/ml carrier salmon sperm DNA, 100 μl of yeast competent cells, and 600 μl of 40% polyethylene glycol (PEG) solution (1X TE, 0.1M LiAc, 40% PEG). The transformation mix was incubated at 30 ^{o}C for 30 min at 250 rpm, followed by the addition of 70 μl dimethyl sufoxide (DMSO) and heating at 42 ^{o}C for 15 min (Matchmaker LexA two-hybrid system and Matchmaker LexA libraries user manual, Clontech, Mountain View, CA). The cells were plated onto glucose based SD media lacking uracil, histidine, tryptophan (SD-U-H-T) and incubated at 30 ^{o}C for 2-3 days until colonies appeared. Glucose based synthetic media was used for normal maintenance of the plasmids in yeast cells. Another synthetically derived media used in this study was galactose/raffinose (SD gal/raf) based media which (Clontech, Mountain View, CA) was used to induce the expression of the library proteins.

E. Isolation of LexA fusion proteins in the yeast cells

Five ml of overnight culture of EGY-48 cells containing the bait (pLexA-γ181) and reporter (p8op-LacZ) plasmids were grown in SD-U-H media, subcultured into fresh SD-U-H media, and grown until they reached mid log phase (OD~ 0.5-0.7). One and a half ml of culture was subjected to centrifugation for 3min at 13000xg and the resultant pellet was resuspended in 500 μl of 50 mM Tris HCl/ 10 mM NaN_3, pH 8.0. Following a

second centrifugation, the pellets were resuspended in 50 μl of 2X SDS sample buffer (containing 2 ml glycerol, 5% SDS, 0.25 M Tris-HCl, 40 μl of 5 mg/ml bromophenol blue, and 10% beta mercapto ethanol/10 ml). Samples were vortexed, boiled for 5 min, and frozen quickly in dry ice or liquid N_2. After adding 50 μl more 2X SDS buffer, cells were centrifuged at 13000xg for 5 min. The supernatant fraction was used for western blotting.

F. Identification of LexA-γ181 fusion protein expression in the EGY48 cells

Whole cell proteins were extracted from the yeast cells containing bait and prey plasmids as mentioned above. After loading the samples onto a 4-20% polyacrylamide gel (Nu Sep gels), the gel was subjected to electrophoresis at 200 V for 1 hour, after which proteins were transferred to a PVDF membrane previously activated in 100% methanol for 15 min. Transfer of proteins at 100V was performed in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol) for 1 hour. Following the transfer, the membrane was blocked for 1 hour at room temperature with blocking solution (5% non fat dry milk powder, Tris buffer saline (TBS; 20 mM Tris base and 137mM sodium chloride, pH 7.6). The blocked membrane was rinsed with TBS for 15 min and then incubated with rabbit polyclonal anti LexA (Millipore, Temecula, CA) antibody (1:20000 dilution in 1% milk/TBS/0.1% tween 20) for 1-2 hours at room temperature. The membrane was washed three times with 1% milk/TBS/0.2% Tween-20 and then incubated with a goat anti-rabbit polyclonal alkaline phosphatase conjugated secondary antibody (1:10,000 dilution in 1% milk/TBS). Again the membrane was washed three times for 10 min each with 1% milk/TBS/0.2% Tween-20. After a quick rinse with TBS,

the membrane was incubated with 1ml of western blue stabilized substrate for alkaline phosphatase (Promega, Madison, WI) until color developed. The resulting image of the developed blot was scanned using a Hewlett-Packard Scanjet 4890.

G. Human fetal brain cDNA library amplification

The human fetal brain cDNA library cloned into pB42 was obtained from Dr. Erica Golemis (Fox Chase Cancer Center). This construct required amplification prior to library screening. Therefore, the vector was electroporated into high efficiency electrocompetent Mega X DH10B T1R *E.coli* cells. Sixty μl of electrocompetent cells were combined with 0.5 μl of pB42-cDNA library plasmid (60 ng/ μl) into pre-chilled 0.1 cm electroporation cuvettes (Fisher Scientific). The cells were electroporated at 2.0KV, 200 ohm, 25 μF by using the BIO-RAD gene pulser II apparatus. Immediately after electroporation, 1 ml of SOC media (2% bacto tryptone, 0.5% bacto yeast extract, 0.2% 5M NaCl, 0.25% 1M KCl, 1% 1M $MgCl_2$, 1% 1M $MgSO_4$, 2% 1M glucose) was added to the electroporated cells. The cells were grown for 1hour. Eighteen μl of cells representing three times the number of independent clones $3.5x10^6$, from this transformation were suspended in 51 ml of LB broth and then the entire mixture was plated on 150 mm LB plates containing 100 μg/ml of ampicillin (300 μl/plate). After incubation of the plates at 32 ^oC for 18 hrs, cells were collected from plates in 4 ml of LB broth by scraping the cells off the plates. Plasmid was isolated from collected cells by using the Qiagen maxi prep plasmid isolation kit (Qiagen, Valencia, CA).

H. Library screening

EGY 48 cells containing bait (pLexA-γ181) and reporter plasmid (p8op-LacZ) were made competent as described above. pB42-cDNA library plasmids were transformed into the competent cells. Approximately 25 transformation reactions were carried out for library screening. For each transformation reaction, 10 μl of pB42-cDNA library plasmid (126 ng/μl), 10 μl of 10 mg/ml carrier DNA, 70 μl of competent cells, and 600 μl of a 40% PEG solution (1M TE, 0.1M LiAc, 40% PEG) were combined. The transformation was carried out at as mentioned above. Cells were plated onto SD-U-H-T plates and then incubated at 30 ^{o}C for 2-3 days until colonies appeared. All cells were pooled into 5 ml of sterile water by gently scraping the colonies off each plate. Collected cells were resuspended in 60% glycerol solution and frozen in 1ml aliquots. One ml of the glycerol stock was diluted in 500 ml of galactose/raffinose lacking uracil, histidine, and tryptophan (SD gal/raf -U-H-T) media and grown at 30 ^{o}C, 250 rpm for 12 hours to induce the library. Approximately 12 SD gal/raf-U-H-T-L plates were plated with 10^{7} cells for library screening. Plates were incubated at 30 ^{o}C for 2-3 days until colonies appeared.

I. Chloroform-agarose overlay assay for detection of potential interactions

Colonies that appeared on SD gal/raf-U-H-T-L plates on day 3 of screening were streaked onto SD gal/raf-U-H-T plates. After incubating the plates for 2-3 days, each plate was overlaid with 10 ml of chloroform. The remaining chloroform was removed, and each plate was overlaid with 7-10 ml of X-gal agarose (1% low melting agarose, 0.1 M $NaHPO_4$ buffer pH 7.0, 0.25 mg/ml X-gal). Plates were incubated at 30 ^{o}C until color

developed.

J. Rescue of library plasmid in the KC-8 bacteria

Plasmid DNA was isolated from the putative positive EGY-48 yeast colonies that showed positive interactions during library screening by using the Zymoprep yeast plasmid miniprep kit II (Zymoresearch, Orange, CA). Isolated plasmid DNA was electroporated into KC8 cells (Clonetech, Mountain View, CA) by using 1 mm gap cuvettes on BIO-RAD gene pulser II set to 1.8 KV, 10 uF, 600 ohm. After electroporation, cells were plated on M9-*TRP* selection media (7.5 mM $(NH_4)_2$ SO_4, 33 mM KH_2PO_4, 60 mM K_2HPO_4, 1.7 mM sodium citrate, 15 g agar, 1M $MgSO_4.7H_2O$, 20ml 20% glucose, 0.74 g URA dropout mix (Clontech, Mountain View, CA), 2 ml 1% thiamine HCl, and 50 μg/ml ampicillin) to rescue the library plasmids. Plates were incubated for 2-3 days at 37 ^oC until colonies grew. Because of the selection pressure the cells effectively retained the library plasmid, but eliminated the bait as well as reporter plasmid.

K. Redundancy determination

To determine any redundancy among the isolated clones the screened library plasmids were digested with EcoRI and XhoI, or the frequent cutter HhaI (New England Biolabs, Baverly, MA). Digested plasmid DNA was loaded onto 0.7% agarose gel (containing 0.7% agarose, 2 μl of ethidium bromide, 1X TBE buffer (0.089 M Tris, 0.089M borate, & 0.002 M EDTA) and then electrophoresed at 100 V for 1hour. The restriction digestion pattern of inserts was compared with a 1kb DNA ladder (New

England Biolabs, Baverly, MA) or identified based on similar digestion patterns. Gel pictures were taken on an Alpha Innotech Fluorochem HD2 system.

L. Sequencing and identification of positive clones

Plasmid DNA was isolated from the putative positive clones and used for sequencing. PCR reactions were carried out in a Techne TC-512 thermocycler by combining 200 ng of plasmid DNA template, Big Dye terminator ready mix v3.1 (Applied Biosystems, Foster City, CA), 5 pM forward primer (CTC TGC TGA GTG GAG ATG C), and sterile water. Cycling was performed for 25 cycles as follows: denaturation at 96 ^{o}C for 30 sec, annealing at 50 ^{o}C for 15 sec, extension at 60 ^{o}C for 4 min. Following amplification, the amplicon DNA was precipitated twice by using 75% isopropanol and dried in a speedvac. Each pellet was dissolved in 20 μl formamide buffer. Sanger's dideoxy sequencing was performed and then samples were loaded on the sequencer (ABI PRISM 3130 Genetic Analyzer). Following the sequencing, the clones were identified by using the publicly available Gen Bank database such as BLASTN and BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

M. Specificity testing

Specificity of the γ181 with its interacting proteins was confirmed by transforming the yeast cells containing the p8op-LacZ reporter plasmid with the following plasmids: pLexA-Phk α (encode the α subunit of Phk), pLexA-Phb N' (encodes the N-terminus of the natural substrate of Phk), pLexA-Phb C' (encodes the c-terminal catalytic domain of the Phk substrate Pb) (Andreeva et al., 2002). Bait

transformed cells were also transformed with each putative positive clone and then grown on SD-U-H-T media at 30^{o}C for 2-3 days. Positive transformants were streaked onto SD gal/raf –U-H-T plates and then assayed for β-galactosidase activity as described above.

N. Quantitative β-galactosidase assay

The Galactostar reporter assay system (Tropix) was used for quantitative measurement of β-galactosidase activity in yeast cell lysates. The Galactostar system is very sensitive due to the use of the chemifluorescent substrate, 1, 2 dioxetane (Applied Biosystems Galacto star system protocol, pp 2-3). Five ml of an overnight yeast culture grown in SD-U-H-T media was subcultured to induce library protein expression. Cells were grown until they reached mid log phase (OD~0.5). One and a half ml of the culture was centrifuged at 12000xg for 0.5 min and resuspended in 1X Z buffer (0.5 M sodium phosphate pH 7.1, 50 mM KCl, 5 mM $MgSO_4$). Cells were again pelleted and then resuspended in 300 μl of 1X Z buffer. Two quick freeze and thaw cycles were carried out for 100 μl of cells in liquid nitrogen. Cells were pelleted by centrifugation at 12,000xg for 5 min at 4 ^{o}C. Reaction buffer mix (300 μl) was added to 20 μl of the supernatant. The reaction buffer mix was prepared by 1:50 dilution of the substrate in reaction buffer diluent (100 mM sodium phosphate pH 7.5, 1 mM $MgCl_2$, and 5% sapphire-II enhancer). The reaction mixture was incubated for 90 min and luminescence was measured by using a TD 20/20 Turner luminometer for 10 sec. Relative light units were recorded for each sample. Data was represented as Modified Miller Units (MMU): the average of the relative light units normalized to the original yeast cell culture concentration (RLU/OD of the culture).

RESULTS

A. Cloning and characterization of γ181 bait

The yeast two-hybrid assay was performed to identify γ181 interacting proteins *in vivo*. Gamma 181, cloned into the pLexA vector, was used as bait, and a human fetal brain cDNA library cloned into the pB42 vector was used as prey in the screening process. To construct the pLexA-γ181 recombinant vector, PCR amplified, digested γ181 product was cloned into precut pLexA vector. Results of the cloning indicated that out of 12 isolated colonies, 11 contain the γ181 insert as confirmed by restriction digestion (Fig. 3.1). The presence of γ181 in the pLexA vector was further verified by sequencing the recombinant pLexA-γ181 plasmid.

Additionally, before starting the actual screening process, the bait fusion protein (LexA-γ181) expression in EGY48 was verified by western blotting (Fig. 3.2). As seen in Fig. 3.2, when probed with an anti-LexA antibody, LexA-γ181 was efficiently expressed in the yeast, equal to or more than that of the positive control LexA-p53 protein. Another protein used in this study, LexA-Bicoid was also efficiently expressed in the yeast cells. In a two-hybrid study, all the proteins must be targeted to the nucleus since transcriptional activity is to be quantified as a report of protein interactions. Even though Brent and Ptasshne, (1984) showed that the LexA protein expressed in yeast is passively transported into the nucleus, verification of bait nuclear localization is necessary. The LexA-γ181 fusion protein nuclear localization was detected by a nuclear repression assay.

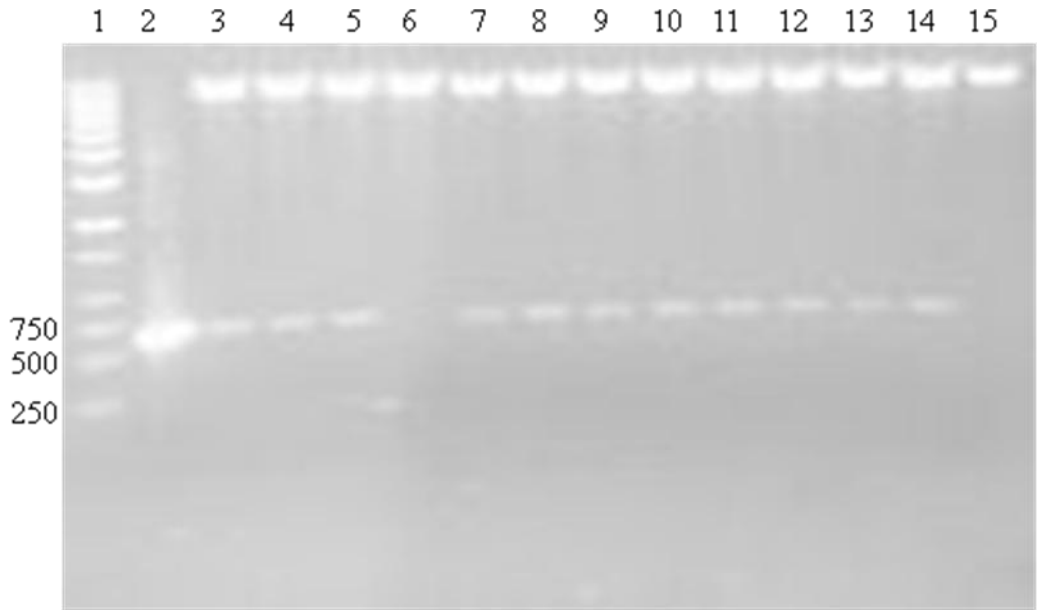

Fig 3.1. Identification of positive clones containing γ181 in pLexA vector. Plasmid DNA was isolated from the positive clones and then digested with EcoRI & BamHI. Size of the γ181 was compared with the 1kb quick load DNA ladder (New England Biolabs). Gel lanes are indicated as 1. Marker, 2. PCR amplification of pUC57-γ181 (positive control), 3-14. Restriction digestion of plasmids isolated from positive clones, 15. Restriction digestion of pLex-A vector (negative control).

Fig. 3.2. Identification of LexA fusion protein expression in yeast cells. Whole yeast cell proteins extracts were probed for LexA fusion proteins using an anti-LexA antibody which recognizes the DNA binding regions of the LexA protein. Immunoblot was performed with the yeast cell lysates containing the following fusion proteins: Lane 1, Marker; Lane 2, LexA-p53; Lane 3 and 5, LexA-γ181; Lane 4, LexA-bicoid. Both the positive control p53 and the γ181 bait are expressed in EGY48 yeast.

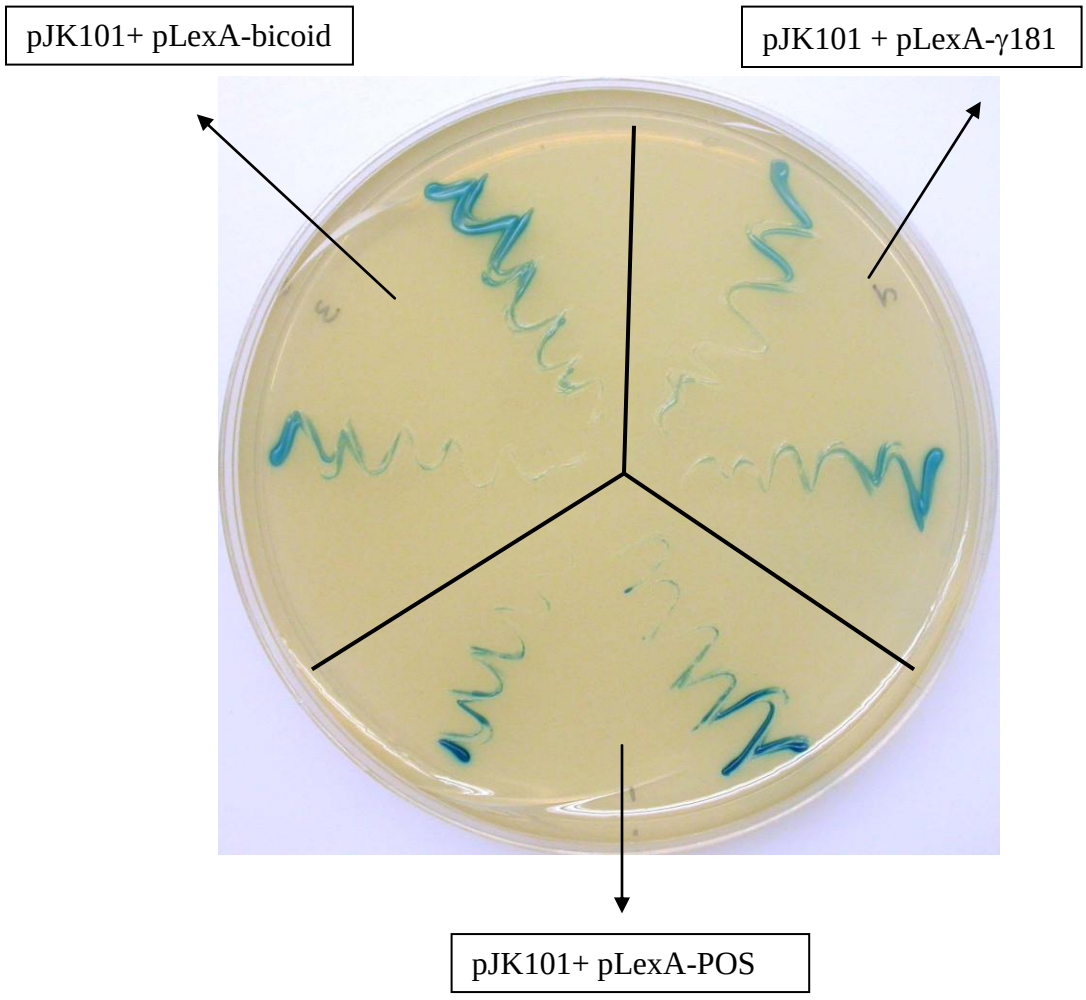

Fig.3.3. Repression assay for nuclear localization of LexA-γ181. Nuclear localization of γ181 was identified by transcriptional repression of the LacZ gene. pLexA-γ181 transformed yeast cells repress the transcription of the reporter gene identified as light blue color in the picture above.

In this assay, LexA fusion proteins bound to the operator located in between the UAS and transcriptional start site inhibited the transcriptional activation of the downstream targets by the enhancers bound to the UAS. pJK101 plasmid has an UAS_G followed by 8 LexA operators, followed by a lacZ coding sequence. Yeast cells were transformed with pJK101, pLexA-γ181, pLexA-POS, or pLexA-bicoid in binary combinations for the repression assay and then transcriptional repression of the LacZ reporter gene was assayed. Nuclear localization of LexA-γ181 repressed the transcription of the reporter gene comparable to the positive control, bicoid, a nonspecific *Drosophila* transcription factor, indicating that LexA-γ181 efficiently localizes to the nucleus (Fig. 3.3). As the yeast two-hybrid system is based upon the reconstitution of a functional transcription factor, checking for the autoactivation of the reporter genes (*LEU2* and LacZ) by the bait protein alone is crucial for this study as it could lead to false positives. Autoactivation of the LexA-*LEU2* gene by LexA-γ181 was tested by growing the yeast transformants containing LexA-γ181 alone or LexA-γ181 with pB42 AD in SD gal/raf-U-H-T-L. Negligible growth was observed by the above transformants indicating that LexA-γ181 lacks the ability to auto activate the LexA-*LEU2* reporter gene (Fig 3.4).

B. Fetal brain cDNA library amplification

A fetal brain cDNA library that represents $3.5x10^6$ independent clones was amplified prior to the screening process. During amplification the library was represented three times the number of independent clones. To get such a high number of independent clones the library was transformed into high efficiency electrocompetent cells DH10B and then plated onto LB-AMP plates. Transformation efficiency was calculated as

$0.9x10^{10}$ CFU/μg. The amplified cDNA library concentration was 1262 ng/μl and its purity (A260/280) was measured to be 1.88.

C. Library screening

To carry out the screening process, EGY48 yeast cells were transformed with the LexA-γ181 bait, the fetal brain cDNA library in pB42AD and the p8OP-LacZ reporter plasmid. Cells were plated on synthetic media lacking leucine for *LEU2* selection. Putative positive interactions were identified by leucine protorophy and further confirmed by their phenotypic expression of β-galactosidase activity on SD gal/raf –U-H-T+Xgal media. Thirty seven putative positive interactions were observed based upon *LEU2* reporter gene expression. Of these 37 putative positive clones, only twenty showed positive interactions for the second reporter gene β-galactosidase (Fig. 3.5). The relative strength of interactions was determined based on the intensity of the blue color within a 24 hour period. Scores were given to each colony with (++++) representing the strongest interactors and (+) being the weakest interactors (Table 2).

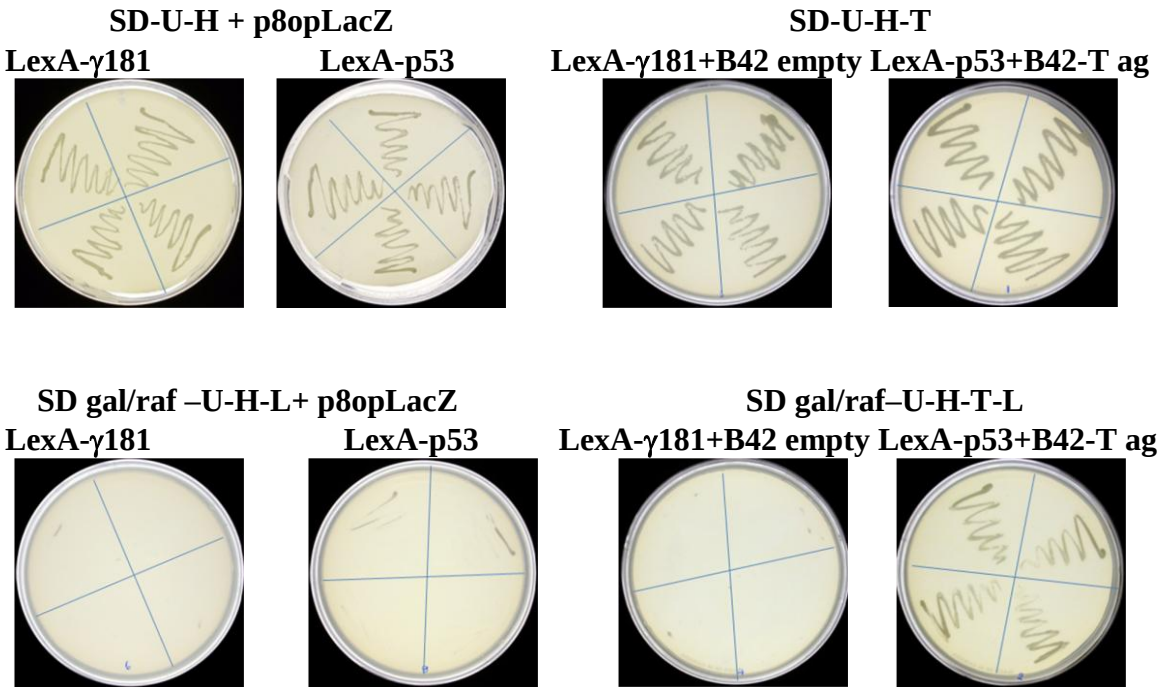

Fig. 3.4. Testing bait γ181 for transcriptional activation of reporter gene *LEU2*. Transcriptional activation of the reporter gene *LEU2* was identified by growing either the LexA-γ181 or LexA-γ181+B42AD transformed EGY48 cells on synthetic growth media (SD-U-H-T) followed by transferring the cells to induction media (SD gal/raf –U-H-T-L) for expression of library proteins. p53 and pB42-Tag vectors were used as control plasmids. p8op-lacZ is the reporter plasmid.

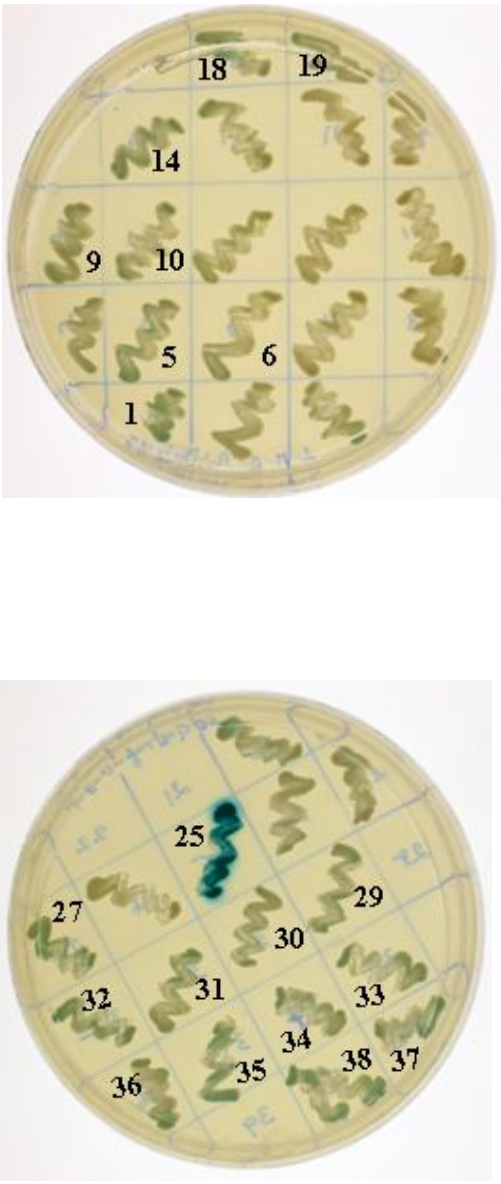

Fig. 3.5. Putative positive interactors of γ181. A total of 37 colonies showed positive interactions for leucine prototrophy. Interactions were further confirmed by the expression of the second reporter gene β-galactosidase. Of those 37 colonies, 20 of them (labeled with numbers) were able to metabolize X-gal in the chloroform agarose overlay assay, as shown above.

S. No	Putative positive clones that are showing positive interactions	Strength of interaction
1	25	++++
2	27, 29, 30, 31, 32, 33, 35, 36, 37, 38	++
3	1, 3, 4, 5, 6, 9, 10, 14, 15, 18, 19, 20, 23	+

Table 2. Phenotypic expression of β-galactosidase by the identified putative positive clones. Putative positive colonies that showed leucine prototrophy were grown in the SD gal/raf-U-H-T selection media and phenotypic expression of the β-galactosidase activity of the individual colony was observed after 24 hours. Clone numbers and the strength of their interactions, (++++) being the strongest, and (+) being the weakest interaction are listed in the table.

In order to isolate the putative library plasmids from the yeast cells, positive clones were grown on SD-*TRP* selection media for two generations to eliminate the bait and reporter plasmids. Subsequently, plasmid DNA was isolated from yeast by using a Zymoprep plasmid isolation kit and then retransformed into the *E. coli* strain KC8 bacteria by electroporation. Transformants were allowed to grow on M9-*TRP* selection media. Following plasmid isolation from the KC8 bacteria, each library clone's insert size was determined by the restriction analysis of the plasmids with EcoRI and XhoI. Based upon comparison to a 1kb DNA ladder, insert sizes ranged from 100-750 base pairs (Fig. 3.6). Redundancy among the clones was also analyzed by digesting the screened library plasmids with the frequent cutter HhaI. Seven nonredundant clones were identified based on the restriction digestion pattern (Fig. 3.7). The unique nonredundant clones were identified as clone 1, 5, 6, 9, 14, 25, and 35. All unique clones were sequenced and then identified by comparision to known sequences in the Gen Bank database by using the BLASTN search engine (Table 3). Of these 7 nonredundant clones, 3 of them were translatable: 1, 5 and 25. Clones 1 and 5 correspond to the ribosomal

proteins L9, and L23, and 25 corresponds to the nuclear antigen 14 (NA14). The clone showing the strongest interaction by β-galactosidase screening showed maximum sequence homology to NA14. Three of the redundant clones 9. 19 and 36 show no sequence homology with any known transcripts or proteins. Column 1 in Table 3 indicates the strength of interactions of these proteins with γ181. Since the truncated γ subunit of Phk has kinase activity, we hypothesize that it may phosphorylate the binding partners of γ181 identified in this screen, such as ribosomal proteins, and NA14. Therefore, by using the PhosK program for predicting high potential phosphorylation sites in proteins (Bloom and Gammeltoft, 1999), we identified high potential serine/threonine phosphorylation sites in the putative γ181 binding partners. RPL9, RPL23 and NA14 all contain anywhere from 2 to 5 high potential phosphorylation sites for serine/threonine kinases (Fig. 3.8).

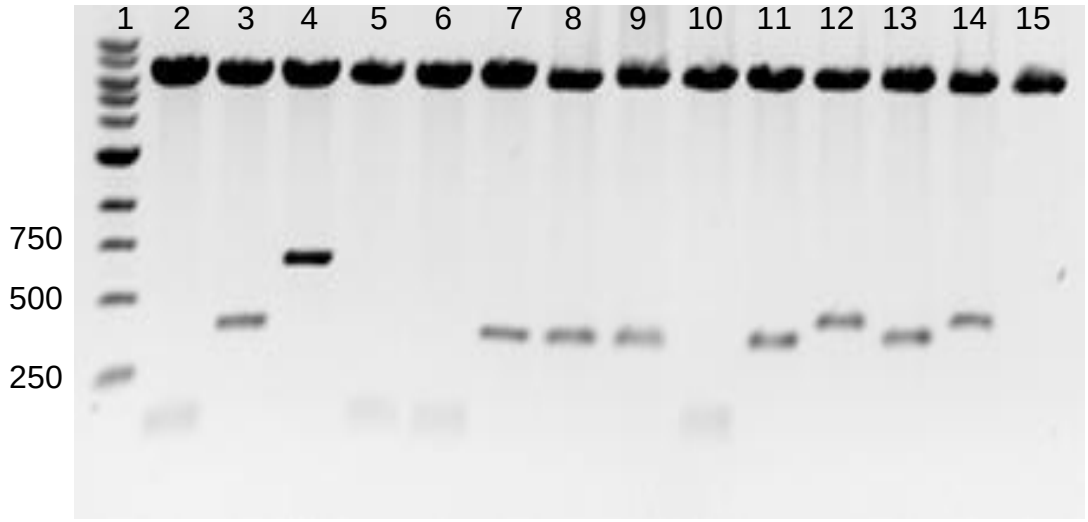

Fig. 3.6. cDNA library insert size determination by restriction digestion. 20 putative positive clones (Lanes 2-14) were digested with the restriction enzymes EcoRI and XhoI. cDNA library insert sizes ranged from 100- 750 bp as observed from the 0.7% agarose gel. Lane 1, NEB 1Kb DNA ladder; Lane 15, negative control pB42 vector.

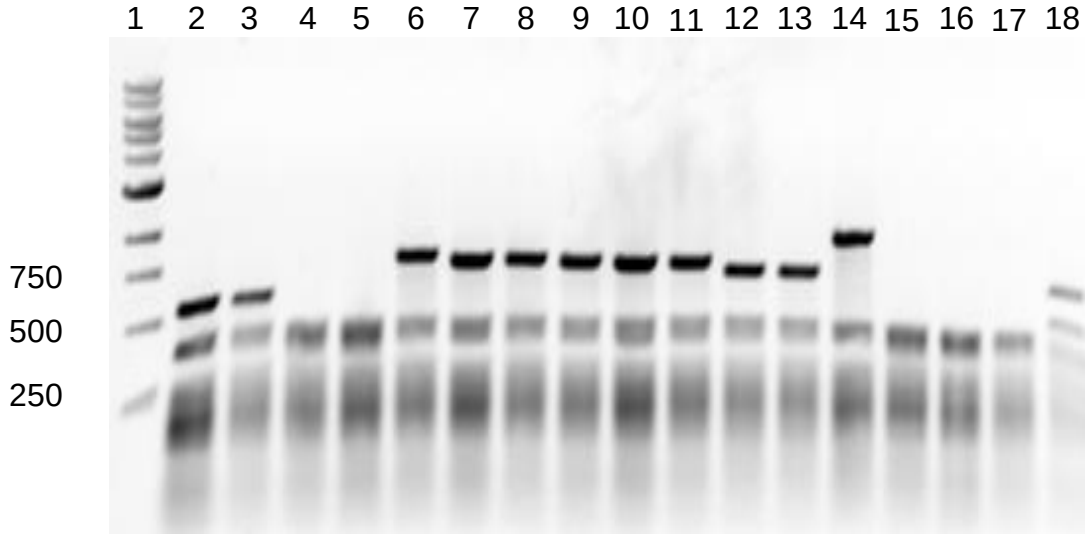

Fig. 3.7. Identification of redundant clones. Redundancy among the clones was identified by digesting the screened library plasmids (Lanes 2-17) with the frequent cutter HhaI. A total of seven unique clones was identified based on restriction digestion pattern as observed in 1% agarose gel. Lane 1, NEB 1Kb DNA ladder; Lane 15, control vector pB42.

Table 3 Identification of isolated clones. Plasmids isolated from the putative positive clones were sequenced and then a BLASTN analysis was performed. The majority of the clones showed maximum sequence homology to ribosomal proteins.

Strength of Interactions	Library plasmid	Transcripts
+	1/1, 5/10	Homo sapiens ribosomal protein L23 **(RPL23)**
++, +, ++, ++, ++	2/5, 7/18, 13/31, 15/33, 19/37	Homo sapiens ribosomal protein L9 **(RPL9)**, transcript variant 1
+,++	6/14, 14/32	Homo sapiens mitochondrial ribosomal protein L12 **(MRPL12)**
+	3/6	Homo sapiens peroxisome proliferator-activated receptor alpha
++	17/35, 20/38	Homo sapiens T-box 3 (**TBX3**), transcript variant 2
++++	9/25	Homo sapiens Sjogren syndrome nuclear autoantigen 1 **(SSNA1)**-aka NA14
+, +, ++	4/9, 8/19, 18/36	No significant similarity found

D. Quantification of LexA-γ181 interactions with the RPL9, RPL23, NA14

The relative strength of the interaction between γ181 and each ribosomal protein and NA14 was measured quantitatively by using 1, 2 dioxetane, a sensitive chemifluorescent substrate for β-galactosidase. To carry out the quantitative assay, prey plasmids containing ribosomal proteins or NA14 coding sequences were retransformed into yeast cells expressing pLexA-γ181 bait plasmid. When the γ181 was assayed against the ribosomal proteins, β-galactosidase activity measured to be 12.0 and 8.8 MMU for RPL23 and RPL 9, respectively. NA14, the strongest interactor with γ181 in the overlay assay, had β-galactosidase activity measuring 10.4 MMU. Interestingly, results obtained in the quantitative assay do not correlate with the overlay assay results. All activities measured are not significantly different when compared with the empty vector control, 10.1 MMU (Fig.3.9). For further verification, the overlay assay was performed again with the retransformed cells. As expected, NA14 again showed very strong interactions with γ181. Whereas the ribosomal proteins interact relatively weakly (Fig. 3.10).

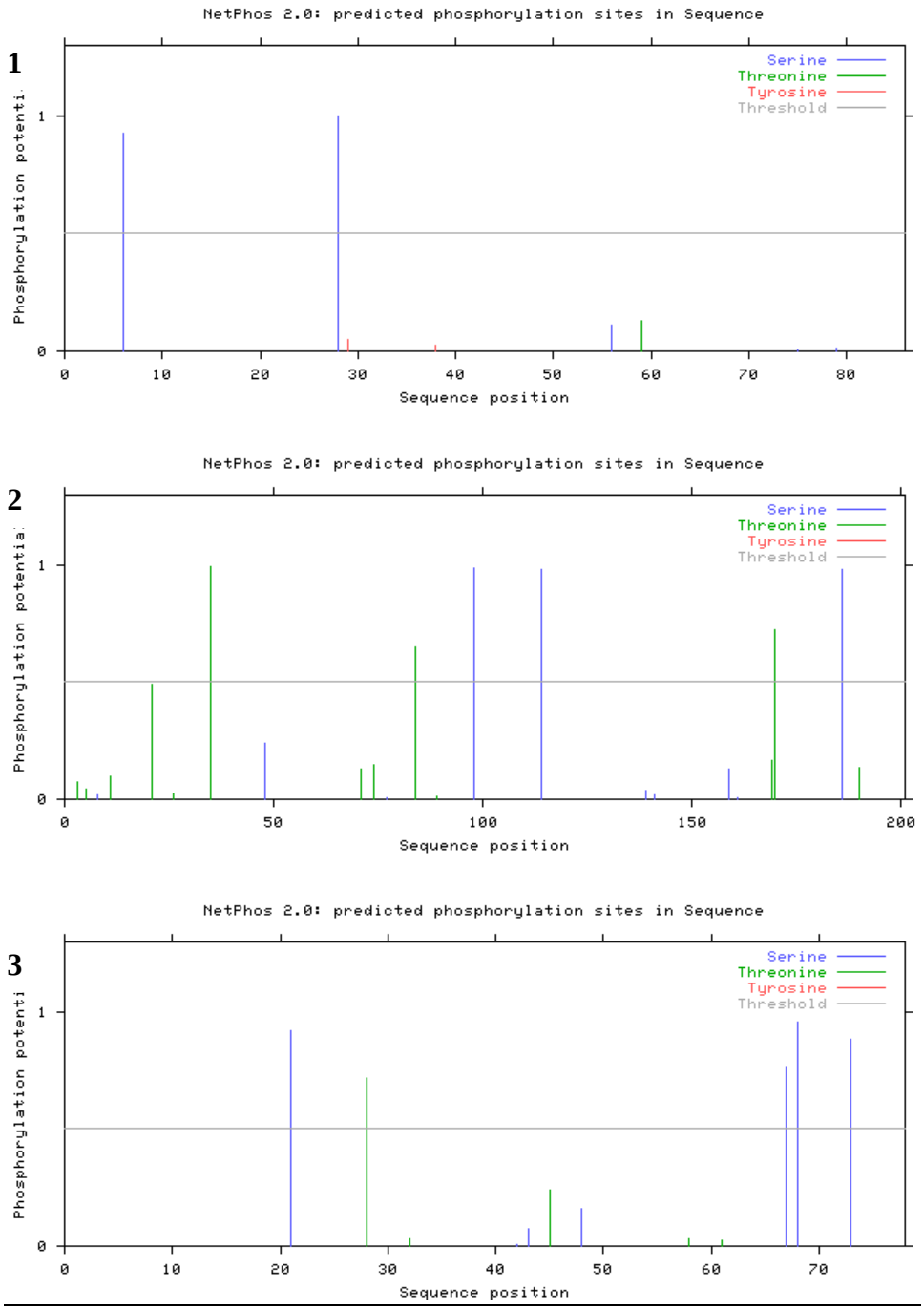

Fig. 3.8 Identification of high serine/threonine phosphorylation sites High potential serine-threonine phosphorylation sites were observed in the γ181 interacting proteins RPL23 (1), RPL9 (2), NA14 (3).

The discrepancy in the data may be due to variability in cell lysis. Yeast cell walls are very tough. Permeabilization of the cell wall and extraction of the whole β-galactosidase enzyme expressed in the cells is very important in the chemiflurorescent reporter assay system. Chloroform was used for permeabilization of the cell walls in the overlay assay, whereas Z buffer (containing sodium phosphate and magnesium sulphate) was used in the chemiflurorescent reporter assay. To eliminate the possibility that the association of γ181 with RPL9, RPL23, and NA14 was due to nonspecific interactions, each protein was tested in binary combinations with three other proteins in the LexA vector: Pb N', the N-terminal regulatory domain of Pb; Pb C', the C-terminal catalytic domain of Pb; and Phk α. Results from this specificity test (Fig. 3.11) revealed that Phk α interacts with RPL23, Pb N' interacts with all three γ181 interactors, and Pb C' interacts with RPl23 alone.

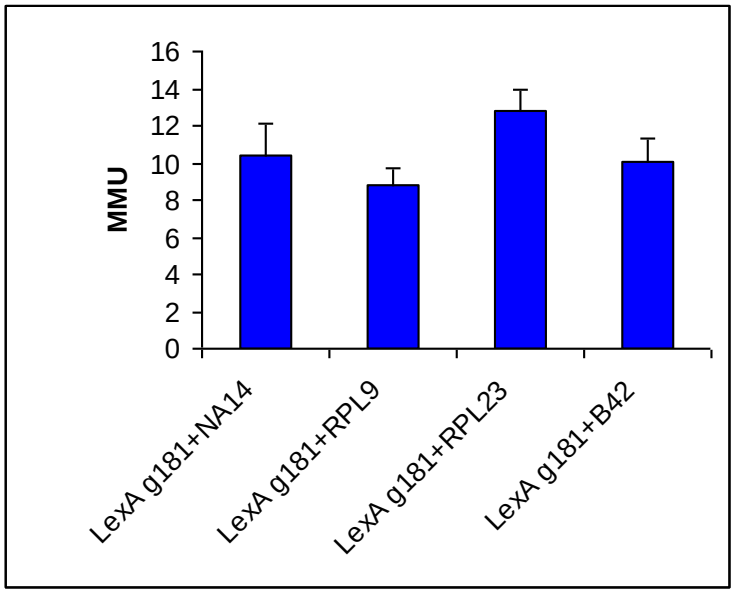

Fig. 3.9. Relative interaction affinities of RPL9, RPL 23, NA14 with γ181. Gamma 181 was assayed with RPL9, RPL23, and NA14. Empty pLexA and pB42 vectors were used as negative controls. Results are expressed as the mean ± S.E.M. of three separate experiments each carried out in duplicate.

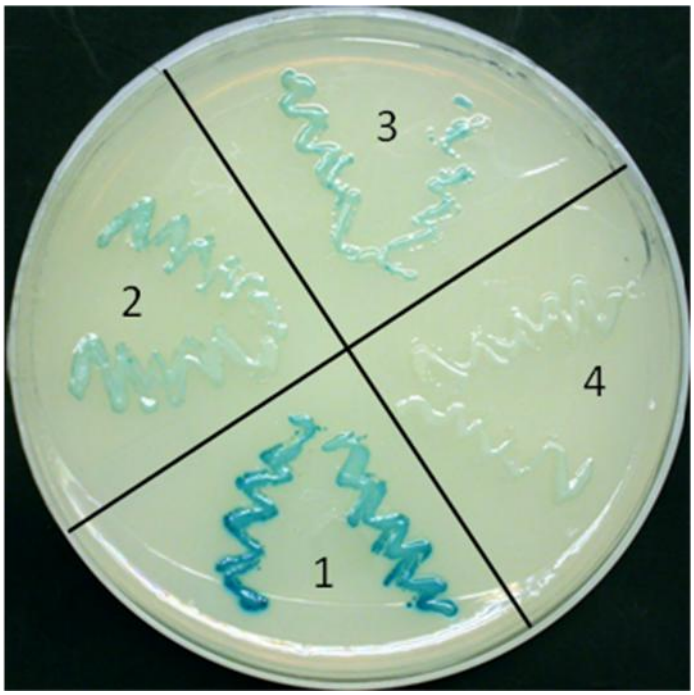

Fig. 3.10. Repeat overlay assay. For reconfirmation of the strength of the interactions, yeast cells containing pLexA-γ181 plasmid were transformed with NA14 (1), RPL23 (2), RPL9 (3), and B42 (4). As shown in the picture, NA14 interacts strongly (1) in the overlay assay. pB42 AD transformed cells (4) do not interact with the γ181 protein.

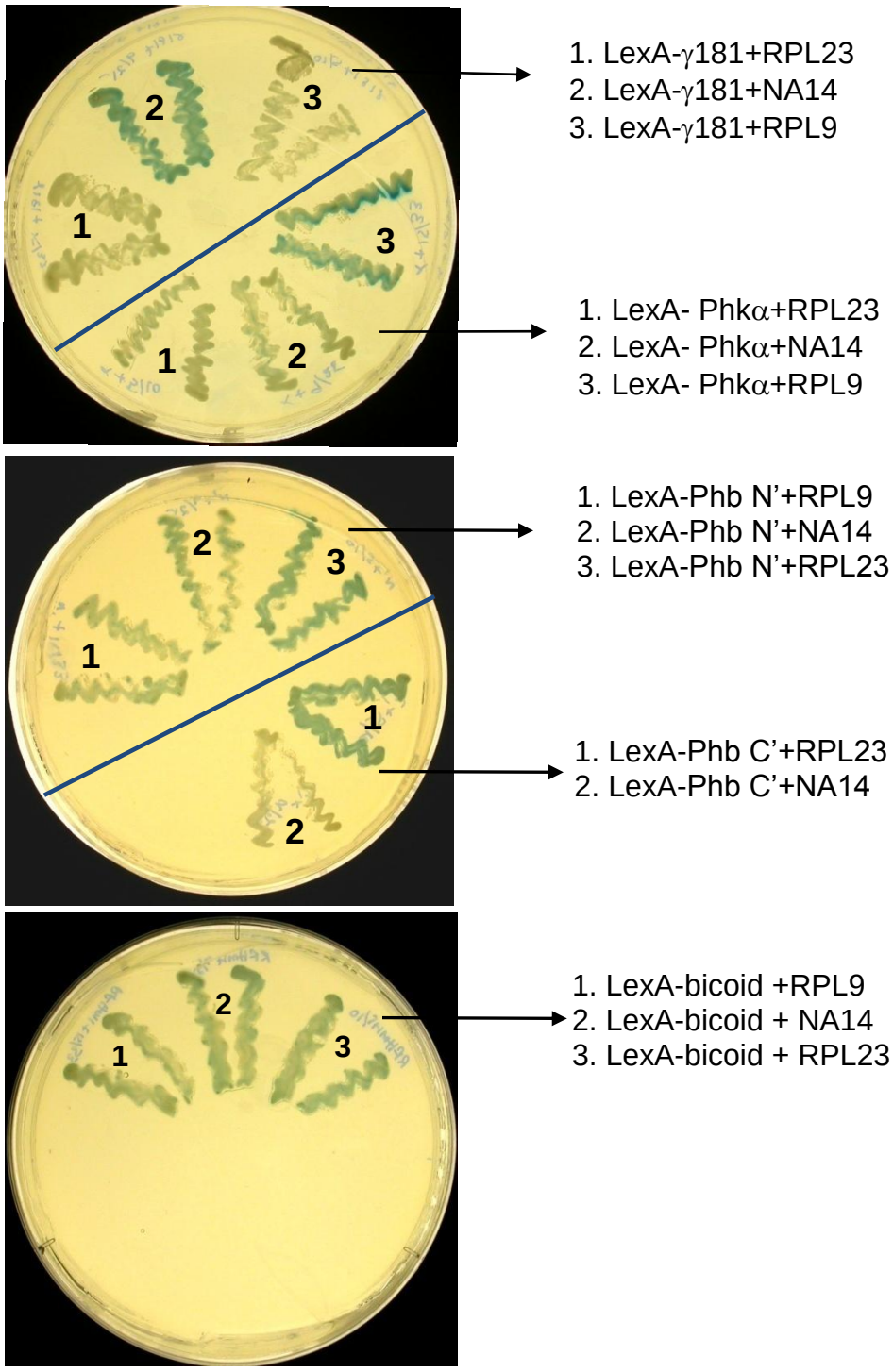

Fig. 3.11. Specificity testing Specificity of the γ181 interactors with the Phk α, Pb N', Pb C, were identified by chloroform overlay assay. As shown above, Pb N' interacts nonspecifically with all three proteins tested, whereas Pb C' interacts with RPL23, and Phk α interacts with RPL9.

DISCUSSION

By using a yeast two-hybrid system we have identified novel interacting proteins of the PHKG1 variant γ181 as NA14, RPL9, and RPL23. NA14 is an autoantigen which is recognized by autoimmune serum from patients with Sjogren's syndrome. Autoantibodies raised against nuclear, cytoplasmic and cell surface components are the most common characteristic features in autoimmune diseases such as systemic lupus, rheumatoid arthritis, and Sjogrens syndrome. Structurally, NA14 consists of a coiled-coil domain and a basic C-terminal domain. Coiled-coil domains are characteristic of other nuclear autoantigens. Immunofluorescence studies revealed that NA14 is localized to the nucleus (Ramos-Morales et al., 1998). Even though NA14 lacks a nuclear localization signal, it is transported into the nucleus either by interacting with another nuclear protein or by passive diffusion because of its small size. NA14 is expressed in almost all tissues, with its expression in testis being very high (Ramos-Morales et al., 1998).

The centrosomal deflagellation inducible protein (DIP13) of *Chlamydomonas reinhardtii* shows 60% sequence homology to NA14. DIP13 is localized to the basal bodies and cytoplasmic microtubules. Because of the sequence similarity of NA14 with DIP13, cellular localization of the NA14 was reexamined using anti-DIP13 antibodies as well as anti-NA14 antibodies, as both of them recognize NA14. Interestingly, immunological studies showed that NA14 localizes to centrosomes and not to the nucleus as previously reported (Pfannenschmid et al., 2002).

The role of the NA14 in the centrosomes is currently unknown. Co-immunoprecipitation and yeast two-hybrid studies showed that NA14 interacts with spastin, a centrosomal protein (Errico et al., 2004). Spastin triggers the depolymerization of the central spindle microtubules by its microtubule severing activity. NA14 acts as an adaptor protein and helps in targeting the spastin to the centrosomes, thus playing an important role in microtubule dynamics (Errico et al., 2004). Posttranslational modifications of NA14 are currently not known. However, PROSITE database analysis revealed that it has consensus motifs for N-glycosylation, a casein kinase II phosphorylation site, and a PKC phosphorylation site (Ramos-Morales et al., 1998).

Another major category of binding partners identified for γ181 were ribosomal proteins. All ribosomes, irrespective of their origin, contain RNA and proteins. Prokaryotes have 70S type ribosomes whereas eukaryotes have 80S ribosomes. The RNA to protein weight ratio is 2:1 and 1:1 for prokaryotes and eukaryotes, respectively (Spirin, 1999). Both large and small subunits of the ribosomes have several different protein components. Proteins in large and small subunits are indicated by L and S respectively followed by a number. Ribosomal proteins are integral components of the ribosomes. Collectively, all of the proteins play a crucial role in the translation process. Assigning function to ribosomal proteins is very difficult, but based on the position of these proteins in the subunit, one can infer function in general terms. The ribosomal protein L9 is critical for ribosome function because anti-RPL9 antibodies significantly reduce protein synthesis (Nag et al., 1991). The crystal structure of the ribosomal protein L9 has been well characterized by X-ray diffraction studies. It has bilobal structure with two ribosomal binding domains that are connected to each other by long α helical coils. Both

domains have conserved aromatic and basic amino acids that likely play an important role in binding to the ribosomes. RPL9 helps in ribosomal assembly and maintains the catalytically active conformation of the RNA molecule (Hoffman et al., 1994, 1996).

In addition to the traditional cell role of ribosomal proteins, a regulatory role for RPL23 has been identified with respect to the transcription factor p53. p53 is a tumor suppressor protein that primarily mediates transcription of several proteins involved in cell growth arrest, apoptosis, and inhibition of angiogenesis, thus protecting the cells from malignancy (Sharpless and DePinho, 2002; Vogelstein et al., 2000). Because of its tumor suppressor function, tight regulation of p53 is very important for normal growth and development of cells. One of the major players that regulates p53 activity in cells is MDM2, a protein which blocks transcriptional activity of p53 (Momand et al., 1992). MDM2 is a ubiquitin ligase and its interacation with p53 initiates the ubiquitination of p53 leading to proteosomal degradation (Kubbutat et al., 1997, Honda et al., 1997). Proper ribosomal assembly and function are very important for normal functioning of cells. Stress on ribosomal biogenesis also regulates the p53-MDM2 feedback loop. In response to ribosomal stress, RPL23 activates p53 by inhibiting its interaction with MDM2 (Dai et al., 2004).

Posttranslational modification of ribosomal proteins is known to occur in reticulocytes and liver cells. *In vivo* phosphorylation of ribosomal proteins in rabbit reticulocytes has been shown by Kabat (1970). Traugh et al., (1993) successfully isolated three different protein kinases from rabbit reticulocytes that phosphorylate proteins of the 40S as well as 60S subunits. Two of the kinases have similar substrate specificity; one is dependent on cAMP for phosphotransferase activity, whereas the other is independent of

cAMP (Traugh et al., 1973). Rabbit liver ribosomal proteins are also phosphorylated by cAMP dependent kinases such as protein kinase I (PKI) and protein kinase II (PKII). PKI is more efficient at phosphorylating the ribosomal proteins than PKII (Eil & Wool, 1973). In this study, we have identified high potential serine/threonine phosphorylation sites in the γ181 interacting proteins RPL9, 23, and NA14 by using the publicly available NetPhosK bioinformatic tool. Biological significance of the posttranslational modifications of ribosomal proteins as well as NA14 is currently unknown.

It should be noted that reports from yeast two-hybrid studies, have shown that ribosomal proteins are quite often identified as false positives. We also observed some weak interactions among ribosomal proteins and γ181. Since the identified interactions were relatively weak, and because RPL9 and RPL23 both interact with many other proteins nonspecifically, it is likely that they are false interactions.

When γ181 was used to screen a human fetal brain cDNA library, we identified 3 major interacting proteins. Those 3 proteins were identified as ribosomal proteins L9, L23 and NA14. The ribosomal proteins, as well as NA14, are unique proteins due to the fact they do not have any known conserved domains. Previous work from our lab has shown that an expressed recombinant γ181 subunit is catalytically active *in vitro* against a synthetic substrate called the S peptide, and its activity depends on phosphorylation by protein kinase C (PK-C) (unpublished data). Based upon our results by NetPhoK predictions, all 3 identified γ181 interactors have potential serine/threonine phosphorylation sites that could be phosphorylated by γ181. Comparision of these sites with the phosphorylation sites of S peptide (SDQEKRKQIS* VRGL) revealed that the γ181 interacting proteins and the S peptide phosphorylatable seryl residue is surrounded

by conserved basic amino acid residues (Fig.4.1). The consensus sequence deduced from the ribosomal proteins and NA14 has similar characteristic features as the *in vitro* substrate S peptide.

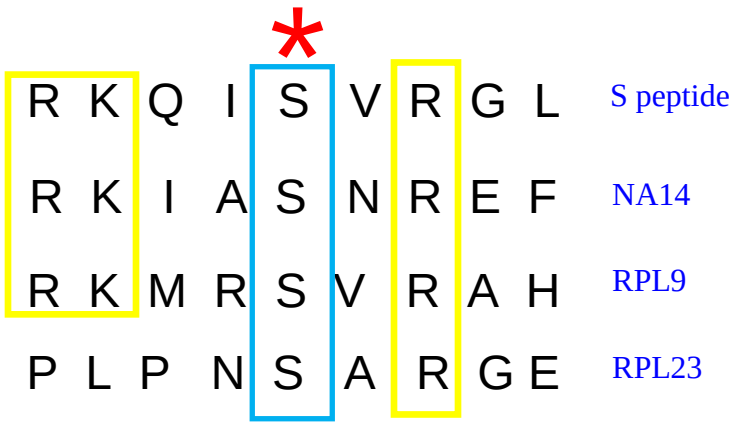

Fig. 4.1. Sequence alignment of high potential phosphorylations sites. High potential phosphorylation sites of γ181 interacting proteins were compared with the artificial substrate S peptide. Basic conserved amino acid residues surrounding the convertible serine residue are marked in yellow boxes.

Furthermore, similarity in the substrate recognition site was observed in the case of the Phk holoenzyme. Phk recognizes the consensus sequence K/R-X-X-S*-V/I-R deduced from its natural substrates such as Pb, glycogen synthase and the β subunit of Phk itself (Pearson & Graves, 1991). Basic amino acid residues surrounding the serine residue are very important in substrate recognition. Arginine at P_{+2} activates Phk (Pickett-Gies and Walsh, 1986 b). Since the amino acids surrounding the serine residue in all three identified proteins show a similar pattern as the *in vitro* substrate S peptide, it is possible these ribosomal proteins and the NA14 are phopshorylated by γ181 *in vivo*.

Comparison of the full length and truncated gamma subunit's structures, as predicted by using the *I-TASSER* folding program (Zhang, 2008, unpublished data),

reveal that γ181 lacks much of the kinase helical domain. As a result, we anticipated that from this study we would potentially identify some binding partners that might compensate for the loss of the helical domain and help in orienting substrates to the catalytic site of γ181. Additionally, since the unique C terminal 21 amino acids of γ181 contains the phosphorylation site for PK-C, we also expected to identify PK-C as an associated protein in our screen. Contrary to these predictions, we did not identify PK-C nor other proteins which may serve as adaptor or subunits in a larger complex. Alternatively, the three proteins that were identified appear to be potential substrates for the enzyme, although again, it is probable that the ribosomal proteins are false positives. Further research is needed to confirm phosphorylation of NA14 by γ181. If γ181 does not phosphorylate NA14, then we need to think about the physiological importance of this interaction in the brain. Previous reports say that NA14 acts as an adapter protein that helps in the localization of spastin to centrosomes. It is possible that interactions between γ181 and NA14 might cause conformational changes in the NA14 resulting in the exposure of known hydrophobic domains that play an important role in its interaction with spastin.

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